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EFFECT OF *Azima tetracantha* ON MITOCHONDRIAL MEMBRANE BOUND ENZYMES IN LIVER ON CARBON TETRACHLORIDE INDUCED OXIDATIVE STRESS IN RATS

Biochemistry

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ABSTRACT

In the present study to investigate the effect of *Azima tetracantha* on membrane bound enzymes in liver on carbon tetrachloride induced oxidative stress in rats. Decrease in activities of Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase were found in CCl_4treated animals. On treatment with different doses (100, 200 and 400mg/ Kg BW) of *Azima tetracantha* leaves extract (ATEE) increased in the activity as dose dependent manner. Among the various doses, 400mg/kg has potential activity than other doses. The silymarin treated rats shows restored the activities of ATPase. The results of the present study indicate that the protective role of ATEE may be related to counteraction of free radicals. Thus, ATEE stimulates the repair of mitochondrial membranes and improve mitochondrial function. These findings suggest that the protective activity of ATEE may indeed play a pivotal role in attenuating free radical damage and stabilize cellular structural integrity.

KEYWORDS

Membrane bound enzymes, Liver, *Azima tetracantha,* Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase

INTRODUCTION:

Membranes are of fundamental importance to cell structure and function. The plasma membrane surrounds the cell, and other membranes form a continuous intracellular surface (endoplasmic reticulum) and the structural basis of intracellular organelles such as mitochondria. Membrane function is vital to many cellular processes including the role of membrane enzymes and receptors in cell growth and signaling. A number of factors are thought to modulate membrane function including dietary components. The dominant lipids in animal cell membranes are phospholipids based on glycerol, such as phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol each with variable fatty acid side chains. The phospholipid bilayer forms the basic structure of all membranes and the presence of a wide range of different proteins confers on membranes a great diversity of function. Cholesterol is found in large amounts in plasma membranes (often equimolar with the phospholipid) whereas endoplasmic reticulum, mitochondrial, and nuclear membranes have a low cholesterol content (Zaachowski, 1993).

Molecular interactions on biomembranes play a prominent role in the communication between cells and in signal transduction pathways. Membrane receptors serve as the main targets able to recognize specific ligands selectively, which can trigger a cascade of functional cell responses. Biological membranes are the first fence that has to be overcome by toxic compounds targeting the cell. One of the most important membrane proteins is adenosinetriphosphathase (ATPase), an integral part of a sodium-potassium pump and the largest protein complex member of P –type family of active cation transport proteins (Skou and Essman, 1992). ATPases play an important role in the maintenance of Ionic gradient by coupling ATP hydrolysis with energy process (Kodama, 1985). ATPases decomposes the adenosine triphosphate into adenosine diphosphate and a free phosphate ion(Gendron et al., 2002; Littleton and Bellen, 1995). Na^+/K^+ATPase is a membrane bound enzyme and inactivation of this enzyme is an important factor in maintaining oxidative stress. Membrane function can either be influenced directly, e.g., by altering fluidity, or indirectly, e.g., by modulation of the free radical-mediated process of membrane lipid peroxidation, which can arise from oxidative stress and result in oxidative membrane damage (Wiseman, 1996).

The rabid metabolic nature of CCl_4 is highly induces the toxicity in liver when it is administrated into living things (De Groot and Noll., 1986; Recknagel, 1989; Clawson, 1989). CCl_4 is bio transformed by the cytochrome P_{450} is a isoenzyme in endoplasmic reticulam to convert CCl_4 into trichloromethyl radical ($CCl_3^{\bullet}$) in the liver after the initiation of lipid peroxidation. $CCl_3^{\bullet}$ reacting with oxygen of cellular proteins and lipids to produce a trichloromethyl peroxyl radical which attacks rabidly lipid membrane of endoplasmic reticulam than trichloromethyl free radical. It has been leads to liver cirrhosis, aging, reduced glutathione, accumulation of triacyl glycerol, Ca^{2+} and Na^{2+} influx and finally cell swelling in mitochondria which allows the mitochondrial membrane damage, , reduced carbonylation of protein , loss of enzyme activity and cell death. These result in changes of structure of the endoplasmic reticulum and other membrane, and loss of glucose-6-phosphatase activation, leading to liver damage. The medicinal value of the chosen plant *Azima tetracantha* leaves has not been extensively worked out. Previously reported that the chosen plant having alkaloids, flavanoids, tannins, cardio glycosides, saponins, and terpenoids like compounds in *Azima tetracantha* (Abirami et al., 2015; Janardhan et al., 2014). Hence in the present study, an attempt has been made to create an animal model with oxidative stress using CCl_4 and the *Azima tetracantha* ethanolic extract (ATEE) on liver mitochondrial membrane Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase activities were evaluated.

MATERIALS AND METHODS

Animals

Male albino rats of Wistar strain approximately 3-4 months young rats (weighing approximately 140-160g) and 24-26 months old rats (weighing approximately 380-410g were used in this study. They were healthy animals procured from Sri Venkateswara enterprises, Bangalore, India. The animals were housed in spacious polypropylene cages bedded with rice husk. The animal room was well ventilated and maintained under standard experimental conditions (Temperature 27±2ºC and 12 hours light / dark cycle) throughout the experimental period. All the animals were fed with standard pellet diet (Gold Mohur, Mumbai, India) and water ad libitum. They were acclimatization to the environment for 1 week prior to experimental use. The experiment was carried out according to the guidelines of the Committee (Ethical No: SAC/IAEC/BC/2016/Ph.D-005) for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Plant Material:

The fresh leaves of *Azima tetracantha* were collected in the month of January 2015 at Melur, Thiruchirappalli District, Tamil Nadu, South India. The leaves were identified and authenticated by Dr. S. John Britto, The Director, the Rabinat Herbarium and centre for molecular systematics, St. Joseph's college Trichy-Tamil Nadu. India. A Voucher specimen (EP001) has been deposited at the Rapinat Herbarium, St. Josephs College, Thiruchirappalli, Tamil Nadu, India.

Preparation of Plant Extract:

Fresh plant material was shade dried and powdered coarsely using electric blender. 250g of dried plant material was soaked in Ethanol for 48 hours. After 48 hrs of soaking the solvent was distilled off under reduced pressure at 50°C and dried in vacuum. The residue was dissolved in isotonic saline and used for the study.

Experimental Design

Body weights of the animals were recorded and they were divided into 4 groups of 6 animals each as follows.

Group I – Normal Rats

Group II – Negative control - Animals were administrated orally with CCl_4(0.5 ml/150g of bw-v/v in olive oil) on 1st, 8th and 16th day.

Group III – Animals were administrated orally with CCl_4(0.5 ml/150 g of bw-v/v in olive oil on 1st, 8th and 16th day) and treated with *Azima tetracantha* leaves extract (100mg/ Kg BW) orally for 21 days.

Group IV - Animals were administrated orally with CCl_4(0.5 ml/150 g of bw-v/v in olive oil on 1st, 8th and 16th day) and treated with *Azima tetracantha* leaves extract (200mg/ Kg BW) orally for 21 days.

Group V –Animals were administrated orally with CCl_4(0.5 ml/150 g of bw-v/v in olive oil on 1st, 8th and 16th day) and treated with *Azima tetracantha* leaves extract (400mg/ Kg BW) orally for 21 days

Group VI –Animals were administrated orally with CCl_4(0.5 ml/150 g of bw-v/v in olive oil on 1st, 8th and 16th day) and treated with Silymarin (20mg/ Kg BW) orally for 21 days

Tissue Homogenate:

Immediately after blood collecting, the animals were sacrificed by cervical dislocation and the liver tissue was dissected out, washed with ice-cold physiological saline. The required amount was weighed and homogenized using a Teflon homogenizer. Tissue homogenate was prepared in 0.1 M Tris HCl buffer (pH 7.4) and used for the estimation of various biochemical parameters. The liver tissue mitochondria were isolated by the method of Johnson and Lardy (1967).

Biochemical assay

The activity of Na+/K ATPase was assayed according to the method of Bonting (1970). The activity of Mg2+ATPase was assayed by the method of Ohniski et al. (1962).. Ca2+ ATPase was estimated according to the method of (Hjerben., et al.1983). The inorganic phosphorus was estimated according to the method of Fiske and Subbarow (1925).

Statistical analysis: Values were expressed as mean SD for six rats in the each group and statistical significant differences between mean values were determined by one way analysis of variance (ANOVA) followed by the Duncan's test for multiple comparisons. The results were considered statistically significant if the p-values were 0.05 or less ($p<0.05$).

Results and Discussion

The total mitochondrial membrane protein content, including both the inner and outer membranes, varies between 60 and 65%, while the inner membrane protein content is believed to be as high as 75%. Because of the high protein content of the inner membrane, it is expected that these proteins are one of the primary targets of mitochondrial-generated ROS. Indeed, membrane protein thiol groups (including ATPases enzymes) suffer extensive oxidation in conditions of Ca^{2+} induced mitochondrial oxidative stress (Andreyev *et al*., 2005). Oxidative damage to cellular membranes plays an important role in the pathobiology of both chronic and acute tissue injury. Unsaturated fatty acids present in the membrane (phospholipids, sterols, glycolipids, and glycerides) and in transmembrane proteins containing oxidizable amino acids, are particularly susceptible to free radical damage (Downey, 1990).

Table 1 and Figure 1 shows the activities of membrane bound ATPases in the liver of control and experimental animals. Decrease in activities of Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase were found in CCl_4 treated animals. On treatment with different doses (100, 200 and 400mg/ Kg BW) of *Azima tetracantha* leaves extract increased in the activity as dose dependent manner. Among the various doses, 400mg/kg has potential activity than other doses. The silymarin treated rats shows restored the activities of ATPase.

Table 1: Effect of plant extract on NA^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase of experimental animals

Groups	NA+K+ATPase (µg of pi liberated/ g tissue/min)	Ca2+ATPase (µg of pi liberated/ g tissue/min)	Mg2+ATPase (µg of pi liberated/ g tissue/min)
Group I	72.0±1.4a	88.8±0.7 a	105.1±0.9 a
Group II	48.0±1.0 b	59.8±0.4 b	78.2±0.6 b
Group III	56.2±0.6 c	68.8±0.7 c	87.8±0.6 c
Group IV	62.6±0.7 d	74.4±0.4 d	92.2±0.4 d
Group V	64.0±0.5 a	83.8±1.4 a	98.2±0.4 a
Group VI	67.8±0.3 a	86.0±0.4 a	102.2±0.4 a

Results were expressed as Mean ± SD for six animals

Mean values within the row followed by different letters (Superscript) are significant ($p< 0.05$) level different from each other and same letter are non-significant were comparison by Duncan's multiple range test (DMRT).

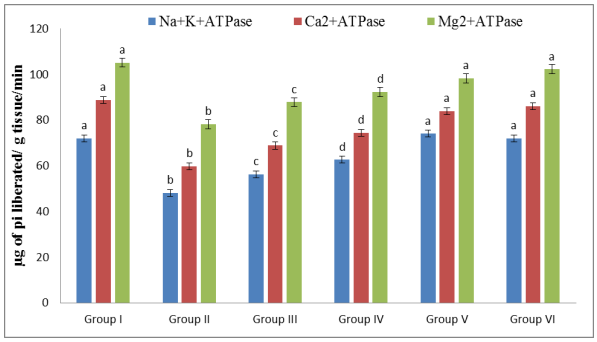

Figure 1: Effect of plant extract on Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase of experimental animals

The activity of the transmembranes enzyme Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase is very susceptible to free radical mediated membrane lipid peroxidation (Mishra *et al*., 1989) and thereby altered the membrane fluidity. Lipid peroxidation has been shown to alter Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase function by modification at specific active sites in a selective manner. Depletion of glutathione and other protective antioxidants may greatly contribute to increasing amount of reactive species, which may also account for impaired activity of Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPas (Qayyum *et al*., 2001).

Oxidative stress is mainly caused by the mitochondrial dysfunction and energy depletion and alteration in the ionic homeostasis leads to loss of cellular integrity and cell death (Anand and Gokulakrishnan, 2012). The activity of mitochondrial Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase in liver was studied after inducing oxidative stress by using CCl_4 as a toxic inducer All the three ATPases showed conspicuous inhibition in CCl_4 induced oxidative stress rats. In *Azima tetracantha* treated rats restored the levels of Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase in liver. Na^+,K^+-ATPase is the protein. The number of organic compounds and inorganic salts, including cardiovascular and anti-cancer drugs, biologically important elements, heavy metals, organic solvents and some toxic organic compounds, such as pesticides and herbicides, strongly modulate enzyme activity on the concentration dependent manner. Because of its high sensitivity to the broad spectrum of toxic compound, as well as potential cardiotonic and anticancer drugs, Na+,K+-ATPase activity can be taken as meaningful index of cellular activity and forms a useful toxicological tool in medicine, pharmacy and environment (Erin *et al*., 1990). In CCl_4 intoxicated rats, the activityies of Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase decreased drastically compared to that of normal group. The activities of Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase recovered significantly ($p< 0.05$) at 100, 200 and 400mg/kg of Azima tetracantha extract compared to that of CCl4 group. In contrast, the Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase activity at 400mg/kg is almost similar to the activity shown by silymarin, a potent membrane protective agent.

This decline of these enzyme activities in CCl_4 induced oxidative stress rat liver mitochondrial membrane is associated with elevated levels of lipid peroxidation products and protein carbonyls were observed our study. These findings clearly suggest that the CCl_4 induced inactivation

of Na^+/K^+ ATPase and Mg^{2+} ATPase in rat membranes is a consequence of oxidative damage (Williamson and Schlegel, 1994).

The regulation of free intracellular calcium (Ca^{2+}) is altered in CCl_4 treated rats, possibly due to reductions in the activity of Ca^{2+} transporters. The plasma membrane Ca^{2+}-ATPase (PMCA) plays a critical role in Ca^{2+} homeostasis, and its kinetic properties change in aged rat. These changes could be due to oxidative modification of PMCA as a result of CCl_4 induced oxidative stresses. Oxidant-induced modifications to Ca^{2+} regulating systems might occur under conditions of oxidative stress and, although not all such modifications necessarily bring about the death of the cell (Beyer, 1994).

In conclusion, CCl_4 intoxicated rats decreased the activity of Na^+/K^+, Ca^{2+} and Mg^{2+} ATPases in liver mitochondrial membrane of rats. Supplementation of different doses (100, 200 and 400mg/ Kg BW) of *Azima tetracantha* leaves extract to CCl_4 treated rats restored the decreased activity of liver Na^+/K^+, Ca^{2+} and Mg^{2+} ATPases in dose dependent manner. Among the various doses, 400mg/kg has potential activity than other doses. The results of the present study indicate that the protective role of ATEE may be related to counteraction of free radicals. Thus, ATEE stimulates the repair of mitochondrial membranes and improve mitochondrial function. These findings suggest that the protective activity of ATEE may indeed play a pivotal role in attenuating free radical damage and stabilize cellular structural integrity.

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